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Process Development of Continuous Glycerolysis in an Immobilized Enzyme-Packed Reactor for Industrial Monoacylglycerol Production

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Continuous and easily operated glycerolysis was studied in different lipase-packed columns to evaluate the most potential process set-ups for industrial monoacylglycerol (MAG) production. Practical design-related issues such as enzyme-filling degree, required reaction time, mass transfer investigations, and capacity and stability of the enzyme were evaluated. A commercially available immobilized *Candida antarctica* lipase B was used to catalyze the glycerolysis reaction between glycerol and sunflower oil dissolved in a binary *tert*-butanol:*tert*-pentanol medium. Considering easy handling of the enzyme and measured expansion when wetted with a reaction mixture, a filling degree of 52 vol % dry enzymes particles per column volume seemed appropriate. Twenty minutes was required to reach equilibrium conditions with a MAG content of 50–55 wt %. Only insignificant indications of mass transfer limitations were observed. Hence, the commercial lipase seemed adequate to use in its available particle size distribution ranging from 300 to 900 μm. A column length-to-diameter ratio of less than 25 did not interfere with the transfer of the fluid mixture through the column. Under the tested conditions, the enzyme could be active for approximately 92 days before enzyme renewal was needed. This corresponds to a very high enzyme capacity with approximately 2000 L pure MAG produced per kg enzyme.

KEYWORDS: ***Candida antarctica*** **lipase B; continuous glycerolysis; enzyme capacity and stability; mass transfer limitations; monoacylglycerols; packed bed reactor**

INTRODUCTION

Lipase-catalyzed glycerolysis processing is of industrial interest since it can be carried out at ambient temperatures and atmospheric pressures (*1–3*). The gentle enzyme technology thereby offers the possibility of large-scale production of heat-sensitive mono- and diacylglycerols (MAG and DAG) with polyunsaturated fatty acid (PUFA) profiles. Well-preserved unsaturated MAG–DAG products, especially purified MAGs, can serve as nutritionally improved food additives and biocompatible emulsifiers and therefore have great potential for new applications in functional foods, pharmaceutical products, etc. (*2–4*).

The use of packed bed reactors (PBRs) is a common strategy to facilitate lipase-catalyzed glycerolysis and other fat interesterification reactions in a continuous and relatively simple way. Various investigators have confirmed the benefits of PBR for lipid modification (*1, 5–8*).

Previously, glycerolysis conducted in a column packed with immobilized lipase was highly potential to ensure a high MAG formation in a short time (*8, 9*). The high efficiency obtained in this system can be attributed to several parameters described below. Glycerol and oil mixing in a binary tertiary alcohol medium reduce the high reactant viscosity and promote improved homogeneity during reactor transit. The solvent acts as an inert carrier material for the reactants to the active site of the enzyme and thus enhances the enzyme activity in the microaqueous systems (*9, 10*). The high-density loading of immobilized enzymes in the PBR facilitates the enzyme contact to the reactant mixture. In addition, the use of lipase bound to a solid carrier material makes separation from product mixture easy and thus facilitates the enzyme reutilization (*4, 11*).

In spite of the improved reactant homogeneity in the solvent glycerolysis system, a heterogeneous reactant mixture with a glycerol-in-oil emulsion occurs (*9*). Hence, the movement of material from phase to phase as well as through the catalyst pores becomes important since it can influence the performance of the immobilized enzyme reactor. Mass transfer investigations of different-sized enzyme particles and varied flow rates, as well as enzyme reactor performance, have been analyzed for different

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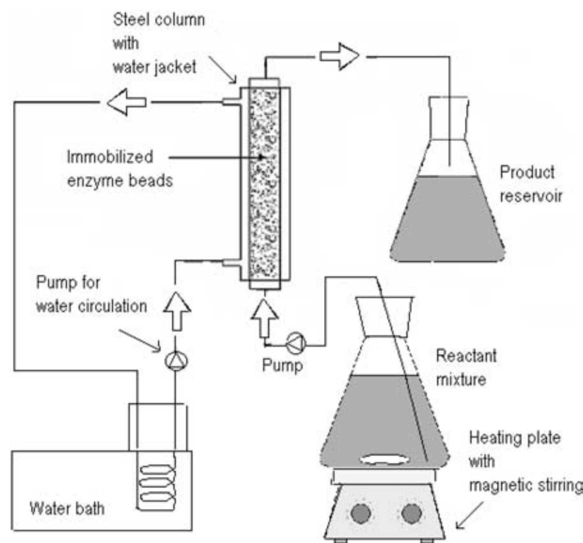

Figure 1. Illustration of the process set-up used for lipase-catalyzed glycerolysis in an enzyme-filled column.

fat interesterification reactions (*1, 5–8, 12*). However, to our knowledge, the available literature lacks investigations that combine these aspects for the glycerolysis process.

The costs of immobilized enzymes are generally high as compared to the costs of traditional chemical catalysts (*4*). Hence, longer use of the enzyme is essential to overcome some of the drawbacks of the increased expenses. Thus, evaluation of the enzyme capacity is crucial to rationalize the performance and operation efficiency.

The aim of this study was to examine the most appropriate process design for an efficient and simple glycerolysis process in an immobilized lipase-packed column reactor for industrial utilization. Practical design-related issues such as required reaction time, enzyme capacity, expansion of the enzyme during wetting, and the effect of different column length-to-diameter ratios, fluid velocities, and particle sizes of the enzymes were evaluated. These investigations were applied to reveal which basic features need to be considered to obtain an industrially beneficial procedure.

MATERIALS AND METHODS

Materials. Sunflower oil containing 97.1% triacylglycerol (TAG), 2.5% DAG, and 0.4% MAG was provided by AarhusKarlshamn AB (Aarhus, Denmark). The predominant fatty acids of the oil were C16:0, 6.7; C18:0, 3.7; C18:1, 26.3; and C18:2, 61.2 (wt %). Novozymes A/S (Bagsværd, Denmark) supplied Novozym435, a commercially available immobilized lipase from *Candida antarctica* lipase B (CALB). The lipase was immobilized by physical adsorption onto an acrylic type macroporous support based on a hydrophobic matrix of polymethyl methacrylate (PMMA) (Lewatit VP OC 1600). Characteristics of the porous spherical beads were as follows: bulk density, 430 kg/m^3; particle diameter, 300–900 μm; surface area, 80–150 m^2/g; and pore diameter, 140–170 10^{-10} m (*13, 14*). Glycerol with a purity of 99.5% w/w was purchased from VWR International Ltd. (Albertslund, Denmark). *tert*-Pentanol (TP) (2-methyl-2-butanol) with a purity of 99% and *tert*-butanol (TB) (2-methyl-2-pentanol) with purity of 99.7% were purchased from Sigma-Aldrich (Brøndby, Denmark).

Continuous Glycerolysis in Enzyme-Packed Column. The process set-up is illustrated in **Figure 1**. Reactant mixtures of sunflower oil, glycerol, and solvent were prepared in a glycerol-to-oil molar ratio of 4–5 and preheated to 50 °C under magnetic stirring. Each mixture, maintained at the set temperature, was pumped upwards through a column by a pump (FMI Laboratory Pump QG150, Micro Laboratory AARHUS A/S, Højbjerg, Denmark). The column consisted of a jacketed steel column packed with the dry enzyme as it was obtained. Unless otherwise stated, the column dimensions were as follows: 20 cm in length and 1.5 and 3.8 cm in inner and outer diameter, respectively. The column temperature was set to 40 °C, maintained by a water bath circulation. Initially, the enzyme bed was wetted and air was removed from the system by running reactant mixture through the column, equivalent to at least four times the reactor volume. To easily implement data into different enzyme dosages, reactor sizes, or flow rates, the following reaction time was used (*15*)

$$\tau = \frac{W}{\nu_0 \times \rho} \tag{1}$$

where τ is the reaction time (min), W is the weight of catalyst pellets on dry matter basis (g), ρ is the bulk density of the dry enzyme g/cm^3, and ν_0 is the volumetric flow rate of the reactant mixture (cm^3/min). Sampling was not started before flow through the enzyme bed equal to four times of the reaction time (τ) was reached. Two samples were hereafter taken and stored at −20 °C prior to analysis by gas chromatography (GC) as described elsewhere (*2*). Results are based on double determinations and are expressed as weight percentages of the product mixture based on glycerol (Gly), MAG, DAG, and TAG, free fatty acid (FFA), and fatty acid ester (FAE) contents.

Screening of Varied Enzyme Loadings in PBR. Glycerolysis with 4, 8, and 12 g of dry enzyme filled in the column was carried out. A reactant mixture of 52.7 wt % TB:TP 80:20 v/v, 33.3 wt % sunflower oil, and 14.0 wt % Gly was used. Different flow rates in the range from 0.24 to 1.9 mL/min were adjusted based on the enzyme amount (4, 8, and 12 g) to obtain reaction times of 5, 10, 15, 20, 30, and 40 min in the column. Down flow was used in this set-up to minimize the risk of back-mixing in the only partially enzyme-filled column.

Measurement of the Expansion Degree during Lipase Wetting. A reactant mixture consisting of 52.7 wt % TB:TP 80:20 v/v, 14.0 wt % Gly, and 33.3 wt % sunflower oil was mixed in a conic flask with magnetic stirring. Exactly 100 mL of the reactant mixture was filled in a measuring cylinder. Ten milliliters of dry immobilized lipase beads was carefully added to the measuring cylinder containing the reactant mixture. After 0.5, 1, and 24 h, the volume of the wetted bead "layer" was measured. The expansion factor was then calculated as the volume of wetted enzyme beads divided by the volume of the dry enzyme beads. Results are expressed as average values of triple determinations.

Screening of Different Reaction Times in PBR. Glycerolysis with 8 g of dry enzyme filled in the column was conducted at different reaction times. A reactant mixture consisting of 51.4 wt % TP, 31.8 wt % sunflower oil, and 16.8 wt % Gly was used. Different flow rates in the range from 0.47 to 1.24 mL/min were adjusted based on the enzyme amount to obtain reaction times of 10, 15, 20, 25, 35, and 40 min in the column.

Operation Stability/Capacity of the Lipase in PBR. Glycerolysis with 8 g of dry enzyme filled in the column was conducted under two conditions. (A) In a pure TP system for 42 days. Here, mixtures of 51.4 wt % TP, 16.8 wt % Gly, and 31.9 wt % sunflower oil were pumped from 5 L flasks through the column at a flow rate of 0.6 mL/min to obtain a reaction time of 30 min. (B) In a binary TB:TP system for 48 days. Here, mixtures of 50.0 wt % TB:TP 80:20 v/v, 17.2 wt % Gly, and 32.8 wt % sunflower oil were pumped through the column at a flow rate of 0.95 mL/min to obtain a reaction time of 20 min.

Glycerolysis with Different Particle Sizes of Enzyme Fractions. Novozym435 was carefully separated by a 500 μm sieve into two fractions. The average particle sizes of the two fractions were then measured with a Mastersizer 2000, Malvern Instruments (Leeds, United Kingdom). The PBR glycerolysis reaction was then performed with 4 g of each enzyme fraction and in the downward flow. A reactant mixture consisting of 52.8 wt % TB:TP 80:20 v/v, 33.3 wt % sunflower oil, and 13.9 wt % Gly was used. Different flow rates in the range from 0.24 to 1.9 mL/min were adjusted based on the enzyme amount to obtain a reaction time of 5, 10, 15, 20, 30, and 40 min in the column. Double determinations were made of samples withdrawn after running four and seven times of the reaction time. The experiments were repeated at reaction times of 20 and 40 min to evaluate the inaccuracy of the method. The reaction rates (r_A) for the two enzyme fractions were calculated as the change in mole MAG per unit time (a molar weight of MAG of 355 g/mol was used for the calculation).

Table 1. Column Dimensions, Enzyme Loadings, Flow Rates, and Reaction Times Used for Glycerolysis in PBR with Different Set-ups as Compared to Calculated Fluid Velocity, Reynolds Number, and Measured MAG Content after Reaction

column dimension			reaction parameters			calculated/measured		
diameter (d) (mm)	length (l) (mm)	volume (V) (cm^3)	enzyme loading (kg/m^3)	reaction time (τ) (min)	flow (mL/min)	fluid velocity (υ) (m/s)	MAG content (wt %)	reaction rate (r_A)
34	500	454.0	220	20	11.60	8.4×10^{-5}	55.6 ± 0.3^a	7.84
21	500	173.2	220	20	4.60	2.2×10^{-4}	52.9 ± 0.4^a	7.46
21	400	138.5	220	20	3.50	1.7×10^{-4}	52.5 ± 0.7^a	7.40
21	200	69.3	220	20	1.80	8.7×10^{-5}	53.9 ± 0.4^a	7.60
15	200	35.3	220	20	0.95	9.0×10^{-5}	50.2 ± 1.5^a	7.08
15	200	35.3	227	20	0.92	8.7×10^{-5}	51.2 ± 2.1^b	7.22
15	200	35.3	227	27	0.69	6.5×10^{-5}	54.0 ± 0.3^b	5.64
15	200	35.3	227	34	0.55	5.2×10^{-5}	56.6 ± 0.2^b	4.69
15	200	35.3	227	41	0.46	4.3×10^{-5}	56.6 ± 0.1^b	3.89
21	200	69.3	173	10	2.80	1.4×10^{-4}	44.8 ± 2.0^b	12.63
21	200	69.3	173	20	1.40	6.7×10^{-5}	50.8 ± 2.1^c	7.16
21	200	69.3	173	31	0.90	4.3×10^{-5}	52.0 ± 1.2^b	4.73
15	400	70.7	173	10	2.85	2.7×10^{-4}	44.7 ± 0.3^b	12.61
15	400	70.7	173	20	1.40	1.3×10^{-4}	49.3 ± 5.3^c	6.95
15	400	70.7	173	30	0.95	9.0×10^{-5}	54.2 ± 1.0^c	5.10

[a] Average values of triple determinations ± standard deviation. [b] Average values of double determinations ± standard deviation. [c] Average values of four determinations (two repeated experiments, each with double determinations) ± standard deviation.

Reactions in PBR with Different Dimensions/Flow Rates. Various flow rates from 0.95 to 11.60 mL/min were tested in five different columns at a constant reaction time of 20 min and constant enzyme loading of 220 kg/m^3 in the column. Lower flow rates from 0.46 to 0.92 mL/min were tested in the smallest column at a constant enzyme loading of 227 kg/m^3. Reaction times of 10, 20, and 30 min were examined in columns with different length-to-diameter ratios but with constant enzyme loading of 173 kg/m^3. Used column dimensions, enzyme loadings, flow rates, and reaction times for each of these set-ups are summarized in **Table 1**. The used reactant mixtures were identical for all set-ups and consisted of 52.8 wt % TB: TP 80:20 v/v, 33.3 wt % sunflower oil, and 13.9 wt % Gly. To characterize the pipe flow through the enzyme particles in each column, calculation of the Reynolds number (Re) was performed as (*16*):

$$\mathrm{Re} = \frac{d_p \times \upsilon \times \rho}{\mu} \quad (2)$$

where d_p is the enzyme particle diameter set to 0.6 mm, υ is the fluid velocity calculated as flow/cross area of the column, and ρ is the fluid density set to 900 kg/ m^3. μ is the fluid viscosity of the reactant mixture measured as 62 mPa s at 25 °C with Viscometer–KR140 (Research Equipment Ltd., London, United Kingdom). The flow was hereafter characterized as turbulent if Re ≥ 4000 or laminar if Re ≤ 2000 (*16*). The fluid moving past single particles was then evaluated by calculating the Sherwood number (Sh) as (*15*):

$$\mathrm{Sh} = \frac{k \times d_p}{D} = 2 + 0.6(\mathrm{Re})^{1/2}(\mathrm{Sc})^{1/3} \quad (3)$$

where k is the mass transfer coefficient and the Schmidt number (Sc) is defined as:

$$\mathrm{Sc} = \frac{\mu}{\rho \times D} \quad (4)$$

with the molecular diffusion coefficient (D) estimated to be 10^{-10} m^2/s.

RESULTS AND DISCUSSION

Effect of Varied Enzyme Loadings in PBR. To determine the most appropriate filling degree of dry enzyme in the column, the effect of three loadings on the MAG formation was tested. Results are shown in **Figure 2**. A medium enzyme dosage of 227 kg dry enzyme/m^3 column, corresponding to 52% of the column volume being filled with dry enzyme beads, resulted in consistent results with increased MAG formation at the prolonged reaction time up to 40 min. A lower or higher enzyme load of 114 and 340 kg/m^3 column, corresponding to filling degrees of 26 and 77 vol %, respectively, led to more inconsistent results.

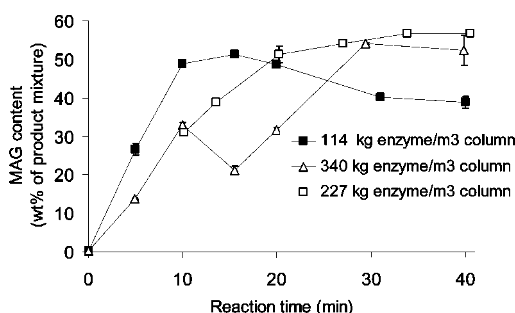

Figure 2. Measured MAG content after glycerolysis at different reaction times in a column filled with varying enzyme loadings (4, 8, and 12 g). Reaction conditions: 52.7 wt % binary TB:TP mixture, glycerol-to-oil molar ratio of 4, reaction temperature of 40 °C, and flow rates from 0.24 to 1.9 mL/min. Error bars represent standard deviations from double determinations.

At the low enzyme load of 114 kg dry enzyme/m^{3}column, the highest achieved MAG content of 51 wt % was obtained after 15 min of reaction and hereafter decreased. This surprising tendency with decreased MAG formation at the prolonged reaction time from 15 to 40 min indicates an unstable set-up. Thus, the low enzyme load somehow resulted in an unevenly reactant–enzyme contact, although downward flow was expected to prevent the risk of channel formation and thereby insufficient contact between reactants and enzyme. For the reaction with high enzyme load, the MAG contents also varied a lot at the different reaction times (**Figure 2**). At the high enzyme load of 340 kg enzyme/m^{3}column, it is plausible that the very tightly packed enzyme layer partly covered the active site of the enzyme. Hence, the reactants might be inaccessible to the active enzyme, thereby leading to reduced efficiency (*5*). Merely an overload of enzyme should be avoided to minimize problems with pressure drop and physical blocking of the column. Therefore, a medium level loading of approximate 227 kg dry enzyme/m^3 column, corresponding to a filling degree of 52 vol %, seemed most suitable for practical operations.

Table 2. Measured Expansion of the Dry Immobilized Lipase (Novozym435) after Wetting with Reactant Mixture for Different Times

time (h)	dry enzyme (mL)	wet enzyme (mL)	expansion (vol %[a])
0.5	10.3 ± 1.0	19.8 ± 2.0	191.9 ± 2.8
1	10.3 ± 1.0	19.8 ± 2.0	191.9 ± 2.8
24	10.3 ± 1.0	19.8 ± 2.0	191.9 ± 2.8

[a] Average values of triple determinations ± standard deviation.

Enzyme Expansion after Wetting with Reactant Mixture. Because the filling degree of 227 kg dry enzyme per m^3 column corresponds to only 52% of the column volume, enzyme swelling during wetting was indicated. The expansion of the immobilized lipase particles was therefore investigated. Results are given in **Table 2**. A quite pronounced expansion degree of 192 vol % of the enzyme–carrier complex was observed as the enzyme changed from dry to wet conditions (**Table 2**). This is in accordance with the literature where the tested enzyme carrier PMMA material swells in some organic solvents (*17, 18*). The observed expansion degree of 192 vol % agrees with a dry enzyme load of 227 kg /m^3 column being the right amount to achieve a total enzyme-filled column after being wetted and stabilized.

A simplified column packing is attractive for practical operations. A full enzyme column is most advantageous to control the flow conditions and optimally utilize the reactor volume. Furthermore, possible back-mixing is avoided and a more complex flow behavior of the fluids does not need to be taken into consideration. Our results show that the time did not influence the expansion degree of the enzyme (**Table 2**). This indicates that swelling saturation of the enzyme is ensured within 30 min. Thus, initial wetting of the dry enzyme bed can easily be done directly in the column in reasonable time before the actual glycerolysis reaction is started. Hence, it is found unnecessary to add glass beads or another filling material in the column to fix the enzyme bed.

Effect of Different Reaction Times. Different reaction times for continuous glycerolysis in the organic medium are evaluated by Yang et al. (*8*) who found a residence time of 30–40 min sufficient to reach maximum MAG yields (*8*). In the actual set-up, a reaction time defined by eq 1 is preferred, since it can easily be used for a solid-catalyzed reaction. To identify the most optimal reaction time based on this equation, reaction times from 10 to 40 min were evaluated.

The time courses are illustrated in **Figure 3**. In general, an increased MAG content was observed with prolonged reaction time reaching the maximum MAG content of 55 wt % after 40 min (**Figure 3**). However, only a minor MAG increase of 5 wt % was obtained as the reaction time was prolonged from 20 to 40 min. Therefore, a reaction time of 20 min should be sufficient to reach an acceptable high MAG formation in accordance with early findings (*9*).

Enzyme capacity or process productivity in terms of MAG content per volume per unit of time is naturally reduced with prolonged reaction time. Hence, the reaction time should be carefully considered to identify the most attractive set-up. The first 10 min led to a MAG content of 31 wt % as compared to only a 19 wt % MAG content increase at the second 10 min (**Figure 3**). A high content of impurities/unwanted side products in the product mixture in general complicates the purification process. Thus, we believe that 20 min will be most beneficial from a practical point of view. Still, a reaction time of 20 min is very short and definitely matches industrial requirements for a rapid and efficient reaction.

Operation Stability and Capacity of the Enzyme in PBR. Long-term continuous glycerolysis was conducted in two different solvent media to investigate the stability and process capacity performance of the lipase for MAG production. The achieved stability calculated with pure MAG produced per hour is illustrated in **Figure 4**, and other parameters characterizing the enzyme performance/productivity are summarized in **Table 3**. In general, the enzyme showed very good stability, capacity, and long half-lives in both solvent systems and maintained activity even after 1000 operation hours.

A higher initial MAG production rate (MAG content/h) was observed in the TB:TP system than that in the TP system, which was most likely ascribed to variations in the reaction parameters (**Figure 4**). Hence, the 50% higher flow rate used in the TB:TP system agrees with the findings of initially 54% more MAG produced per hour. In both systems, the reaction was very fast and had reached equilibrium (50–55 wt %) without effects by the different flow rates.

A considerably more stable TP system was observed in contrast to a clear linear time-dependent decay in the TB:TP system (**Figure 4**). This resulted in remarkably different estimated residual activities of 55 and 90% for the TB:TP and the TP systems, respectively (**Table 3**). Comparison of MAG produced in the two systems at residual activity of 90% indicated a surprisingly higher capacity in the TP system with 25% more MAG produced even with the higher productivity per hour in the TB:TP system considered (**Table 3**). TP only differs from TB by one more carbon atom placed in the alcohol chain (*8*). This certainly leads to a slight difference of polarity, which might bring some difference of the enzyme activity. However, such effects were not noticed in early studies (*8*).

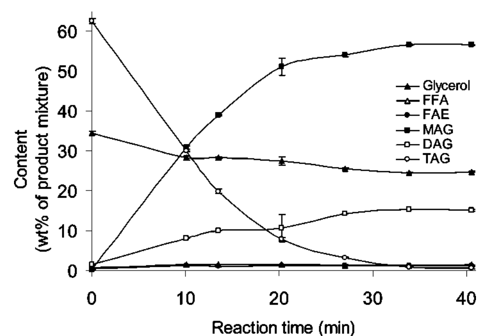

Figure 3. Measured product distribution after glycerolysis in an enzyme-filled column at varying reaction times. Reaction conditions: 51.4 wt % TP, glycerol-to-oil molar ratio of 5, reaction temperature of 40 °C, and flow rates from 0.47 to 1.24 mL/min. Error bars represent standard deviations from double determinations.

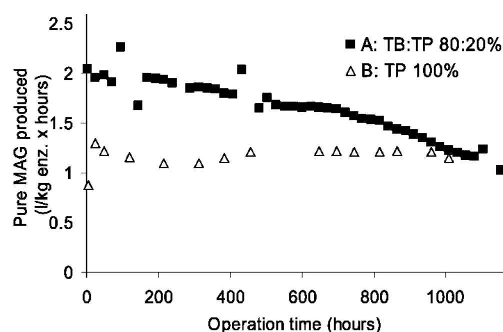

Figure 4. Time course for MAG production per hour after long-term continuous glycerolysis in (**A**) TB:TP 80:20 v/v system (50 wt %) and (**B**) pure TP system (51.4 wt %). Conditions for (**A**): a reaction time of 20 min, a flow rate of 0.95 mL/min, and 48 running days. In (**B**): a reaction time of 30 min, a flow rate of 0.60 mL/min, and 42 running days. Other reaction parameters: glycerol-to-oil molar ratio 5, 40 °C, and 8 g of enzyme.

Table 3. Different Calculated Parameters Based on Results Obtained from Long-Term Continuous Glycerolysis in a TB:TP 80:20 v/v and a Pure TP System

parameter	unit	TB:TP system	TP system
operation time	h	1152	1008
amount used reactant mixture	L	65.7	36.3
total MAG produced[a]	L pure MAG/kg enzyme	1225	1100
final residual activity[b]	%	55	90
reactant exposure at 90% residual enzyme activity[c]	L reactant mixture /kg enzyme	3600	4500
total MAG produced at 90% residual enzyme activity[c]	L pure MAG/kg enzyme	890	1100

[a] Calculated as the area under the curves in **Figure 4** for the entire operation period. [b] Calculated as measured MAG content after ended operation time divided with initial MAG content (set to 100%). [c] The residual enzyme activity was based on the measured MAG content with initially MAG content set to 100%.

Typically, enzymes gradually get less active over time due to deactivation/inhibition. Both systems were exposed to identical temperature, similar operation time, same batch of enzyme, and substrate mixtures with the same source of solvent and Gly, as well as both running in laminar flows. Therefore, similar lipase-inhibiting effects were expected over time such as conformational changes, water deprivation from the enzyme, release of the carrier bonded to the enzyme, disruption in the reactant mixture, etc. (*19, 20*).

The high total reactant mixture exposure in the TP:TB system as compared to the TP system was the only difference that could have influence on the enzyme stability after all of the diagnoses. This indicates a certain exposure limit of the reactant mixture before a decrease in the enzyme activity occurs. Exposure limits of 28.7 and 36.3 L substrate/kg enzyme were determined for the TB:TP and TP systems, respectively, before a significant drop in the MAG production was detected.

In spite of the difference of stabilities in the two organic media (**Figure 4**), deactivation will certainly happen over time, explaining the need for catalyst replacement during the processing. Assuming that the enzyme activity in the TB:TP system follows the common first-order deactivation kinetics, the life time of the enzyme can be estimated from the following equation:

$$A = A_0 \times \exp^{-kd \times \tau} \quad (5)$$

where A and A_0 are enzyme activity at time τ and 0, respectively, and k_d is the deactivation constant. With a half-life set to 1200 h (based on estimation from **Figure 4**) and the lower limit for enzyme usage set to 25% residual activity, the enzyme life time is calculated to 2200 h (92 days). This corresponds to a total productivity roughly calculated as 2000 L pure MAG/kg enzyme. This makes the enzyme attractive for use in commercial plants and offers high economical potentiality for industrial implementation.

Effect of Different Particle Sizes of Enzyme. It has been established that the selection of support material and immobilization method is important for the reaction efficiency of enzymes (*5*). Previous experiments have already confirmed that the hydrophobic PMMA carrier material is beneficial as compared to many other carrier materials for lipase-catalyzed glycerolysis (*8, 21*). However, it is unclear how the particle size of this catalyst affects the reaction efficiency. To address this, pore diffusion resistance was evaluated by conducting continuous glycerolysis with different particle sizes of catalyst beads.

The time courses with average enzyme particle sizes of 388 and 548 μm are illustrated in **Figure 5**, and the calculated reaction rates are shown in **Table 4**. Some differences in the MAG content were observed (**Figure 5**). The initial reaction rate was 30% higher for the 5 min reaction performed with the smaller particles as compared to the larger particles (**Table 4**).

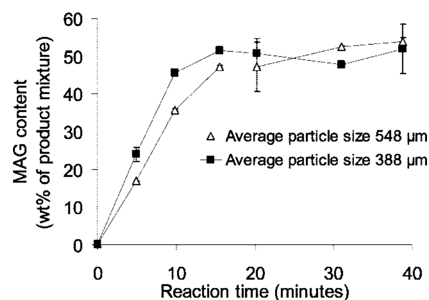

Figure 5. Measured MAG content after glycerolysis in a column filled with different-sized enzyme particles. Reaction conditions: 52.8 wt % binary TB:TP mixture, glycerol-to-oil molar ratio of 4, 40 °C, 4 g of enzyme, and flow rates from 0.24 to 1.9 mL/min. Error bars represent standard deviations.

Table 4. Calculated Reaction Rate (r_A) for MAG Formation Based on Glycerolysis Performed with Different Sizes of Enzyme Particles

time (min)	reaction rate (mmol MAG /min)[a] average particle 388 μm	average particle 548 μm	$r_{A388\mu m}/r_{A548\mu m}$
5	13.70	9.59	1.43
10	13.05	10.23	1.28
16	9.30	8.52	1.09
20	7.03	6.54	1.07
31	4.32	4.74	0.91
39	3.75	3.89	0.96

The difference of initial reaction rates indicates the existence of internal mass transfer limitations for the large particles used. Our observation is in accordance with the findings by Murty et al. for other lipase particles used in the continuous PBR (*1*).

However, the influence of the particle size was not significant in general (**Figure 5**). The reaction rate also converged as the reaction times were prolonged (**Table 4**). If 20 min is selected for the aimed reaction time as previously discussed, the difference of MAG content (**Figure 5**) as well as reaction rate (**Table 4**) is not significantly marginal. Therefore, differences between small and large particles are believed to be only minor in an overall consideration. Furthermore, the reduction of particle sizes of enzyme will increase the pressure drop in principle. This will certainly not be favorable for the process design. With these considerations in mind, internal mass transfer limitations with strong diffusion resistance through the porous particles are neglected. Thus, the lipase is believed to be adequate to use as it is with its commercially available size distribution ranging from 300 to 900 μm.

Effect of Different Flow Rates/Column Dimensions. Studies of oil hydrolysis in immobilized lipase PBR have shown that the reaction rate is affected by the linear fluid flow rate (*1*). Hence, external mass transfer limitations with poor transfer of the reactant mixture to the outer surface of the enzyme particles and poor product transport away from the particle

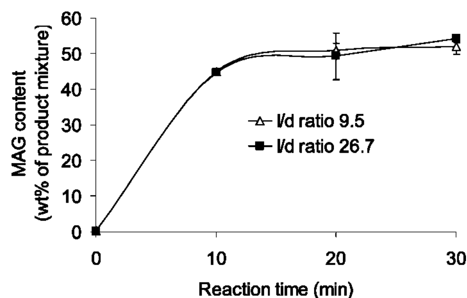

Figure 6. Measured MAG content after glycerolysis in columns with varied length-to-diameter (*l*/*d*) ratios. Reaction conditions: solvent dosage, 52.8 wt % TB:TP mixture; glycerol-to-oil molar ratio of 4; and temperature, 40 °C. In *l*/*d* 9.5: column diameter, 21 mm; column length, 200 mm; flow rates from 0.9 to 5.6 mL/min; and enzyme amount, 12.0 g. In *l*/*d* 26.7: column diameter, 15 mm; column length, 400 mm; flow rates from 0.9 to 5.8 mL/min; and 12.3 g of enzymes. Error bars represent standard deviations.

Table 5. Calculated Sherwood Number for Glycerolysis Performed in Two Columns with Different Length-to-Diameter Ratios

reaction time (min)	*l*/*d* ratio 9.5	*l*/*d* ratio 26.7	difference %
10	3.85	4.57	15.7
20	3.28	3.78	13.3
30	3.03	3.48	13.2

surface into the bulk phase are plausible in the actual set-up. In general, external mass transfer limitations are reduced in PBR by increased flow rates or reduced reactor length-to-diameter ratios, eventually leading to a higher linear flow rate (*5*). Thus, clarification of the effect of glycerolysis conducted with different flow rates and column dimensions was evaluated. The MAG formation and reaction rate under different conditions are summarized in **Table 1**. The MAG content achieved from glycerolysis in a long thin column as compared to a shorter thicker column is illustrated in **Figure 6**, and the corresponding calculated Sherwood numbers are shown in **Table 5**.

Very low Reynolds numbers of less than 100 were calculated for all experiments indicating a laminar flow behavior (Re $<$ 2000). Hence, the flow seems to be dominated by viscous forces with, in theory, uniform nonturbulent flow in parallel layers with little mixing between layers (*16*). A certain degree of viscous forces being dominant for the flow dynamics is in good agreement with the high viscous oil and glycerol raw material although the presence of solvent indeed has reduced the viscosity of the reactant mixture (measured to be 62 mPa s at 25 °C).

At 20 min reaction, similar reaction rates were observed (approximately 7.3 $\pm$ 0.3 mmol/min), independent of the different fluid velocities tested (**Table 1**). This indicates that even low fluid velocities are not critical for the MAG formation and that the transfer of the reactant mixture to the enzyme is not a limiting factor for the process. The fluid velocities yielding laminar flow apparently caused an evenly distributed reaction mixture to the enzyme layer.

Almost identical time courses with similar MAG formations were observed for the two columns with different length-to-diameter ratios (**Figure 6**). Some differences were observed in the calculated Sherwood number with higher values in the long thin column as compared to the shorter thicker column (**Table 5**). Although this indicates a higher external mass transfer resistance in the long thin column as compared to the shorter thicker column, these differences are believed to be negligible. The reason for this is that a Sherwood number of the same order of magnitude for the two different columns can be treated as an insignificant difference. Hence, it seems unnecessary to consider alteration of the column reactor length-to-diameter ratios to dramatically improve the transfer of the fluid mixture through the column.

On the basis of our results, a scaled-up continuous glycerolysis process should not have considerably strong external mass transfer problems within the range of the length-to-diameter ratios used in this study. In a scaled-up reactor with increased dimensions, higher flow rates are used to maintain similar fluid velocity. Thus, it should be kept in mind that higher flow rates can increase the pressure drop and thereby the risk for pump malfunctions, enzyme bed compression, blockage, etc. (*8*). However, the set-up with laminar upward flow pattern and regularly shaped particles is believed beneficial for reducing pressure drop problems (*5*). In addition, calculated pressure drops of less than 1 bar per m/h for glycerolysis confirm minimized risk to encounter pressure drop problems in practical large-scale operations (*8*).

In conclusion, our results showed no foreseen difficulties in establishing an efficient and continuous glycerolysis reaction in an enzyme-packed reactor with respect to industrial operations. Simple dry enzyme packing of the column, no distinct mass transfer limitations, high capacity, and long-lasting activity of the enzyme makes the PBR set-up very suitable for scale-up processing without any obvious problems. Thus, the fulfillment of an easy and practical operation in combination with high reaction efficiency clearly demonstrates the great potential for future implementation of this process in industrial usage.

ABBREVIATIONS USED

CALB, *Candida antartica* lipase B; DAG, diacylglycerol; FAE, fatty acid esters; FFA, free fatty acids; GC, gas chromatography; Gly, glycerol; MAG, monoacylglycerol; PBR, packed bed reactor; PMMA, polymethyl methacrylate; PUFA, polyunsaturated fatty acids; TAG, triacylglycerols; TB, *tert*-butanol; and TP, *tert*-pentanol.

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